

# Rapid, transient induction of ER stress in the liver and kidney after acute exposure to heavy metal: Evidence from transgenic sensor mice

Nobuhiko Hiramatsu<sup>a</sup>, Ayumi Kasai<sup>a</sup>, Shuqi Du<sup>b,c</sup>, Masayuki Takeda<sup>b</sup>, Kunihiro Hayakawa<sup>a</sup>, Maro Okamura<sup>a</sup>, Jian Yao<sup>a</sup>, Masanori Kitamura<sup>a,\*</sup>

<sup>a</sup> Department of Molecular Signaling, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Chuo, Yamanashi 409-3898, Japan

<sup>b</sup> Department of Urology, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Chuo, Yamanashi, Japan

<sup>c</sup> Department of Urology, First Affiliated Hospital of China Medical University, Shenyang 110001, China

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**Abstract** Endoplasmic reticulum (ER) stress-responsive alkaline phosphatase (ES-TRAP) serves as a sensitive indicator for ER stress. In response to heavy metals including cadmium, nickel and cobalt, hepatocytes and renal tubular cells expressing ES-TRAP exhibited ER stress and decreased ES-TRAP activity. In ES-TRAP transgenic mice, acute exposure to cadmium showed rapid, transient decreases in the activity of serum ES-TRAP. It was inversely correlated with the induction of endogenous ER stress markers in the liver and kidney. Our result provides first evidence for the acute, reversible induction of ER stress in vivo after exposure to heavy metal.

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**Keywords:** Endoplasmic reticulum (ER) stress; ER stress-responsive alkaline phosphatase (ES-TRAP); Transgenic mouse; Heavy metal; Biosensor

## 1. Introduction

Heavy metals play essential roles in mediating enzymatic reactions as co-factors. Despite their inevitable roles in biological systems, excessive ingestion of heavy metals causes tissue injury. For example, cadmium is one of the most cumulative heavy metals with an estimated half-life of more than 15 years. Exposure to cadmium via drinking water, foods and cigarette smoke causes its accumulation in a variety of organs, especially in the liver and kidney, the main targets of the cadmium-induced toxicity [1,2].

Endoplasmic reticulum (ER) stress is involved in a wide range of pathological situations including hypoxia and ischemia, viral infection, neurodegenerative disorders, drug-induced tissue injury and metabolic diseases such as diabetes mellitus [3,4]. Previous reports suggested a possibility that, in vitro, some heavy metals may induce ER stress and contribute to

damage of neuronal and glial cells [5,6]. However, currently, little is known about whether and how ER stress is induced in vivo following systemic exposure to heavy metals.

We recently reported that ER stress-responsive alkaline phosphatase (ES-TRAP) is a sensitive, quantitative biomarker for ER stress [7]. In vitro, activity of ES-TRAP secreted by transfected cells is rapidly down-regulated in response to ER stress independent of transcriptional regulation. This phenomenon is observed in a wide range of cell types triggered by various ER stress inducers with high sensitivity and selectivity [7]. In the present investigation, we generated transgenic mice systemically expressing ES-TRAP and used for real-time monitoring of ER stress after acute exposure to heavy metal. Our result evidenced rapid, transient and reversible induction of ER stress in vivo after systemic exposure to cadmium.

## 2. Materials and methods

### 2.1. Cells and exposure to heavy metals

The rat renal proximal tubular cell line NRK52E and the mouse hepatoma cell line Hepa-1c1c7 were purchased from American Type Culture Collection (Manassas, VA). NRK52E cells and Hepa-1c1c7 cells were stably transfected with pSEAP2-Control (BD Biosciences, Palo Alto, CA) encoding ES-TRAP (also called secreted alkaline phosphatase) under the control of the simian virus 40 promoter, and NRK/ES-TRAP cells and Hepa1/ES-TRAP cells were established. These cells were treated with cadmium chloride (25–75  $\mu$ M; Wako Pure Chemical Industries, Osaka, Japan), nickel acetate (500–1000  $\mu$ M; Wako), cobalt chloride (250–1000  $\mu$ M; Wako) or tunicamycin (1  $\mu$ g/ml; Sigma–Aldrich Japan, Tokyo, Japan) for 6 h, and the cells and culture media were subjected to Northern blot analysis and ES-TRAP assay, respectively. All experiments were performed in the presence of 1% fetal bovine serum.

### 2.2. Northern blot analysis

Total RNA was extracted by the single-step method, and Northern blot analysis was performed as described before [7]. cDNAs for 78 kDa glucose-regulated protein (GRP78) [8] and CCAAT/enhancer-binding protein-homologous protein (CHOP) [9] were used for preparation of radio-labeled probes. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

### 2.3. ES-TRAP assay

Activity of ES-TRAP was evaluated by chemiluminescent assay using Great EscApe SEAP Detection Kit (BD Biosciences) [10]. To examine direct influences of heavy metals on the activity of ES-TRAP, recombinant ES-TRAP protein was added with cadmium chloride, nickel acetate or cobalt chloride at several different concentrations, left at 37 °C for 6 h, and its activity was re-tested.

\*Corresponding author. Fax: +81 55 273 8054.

E-mail address: masanori@yamanashi.ac.jp (M. Kitamura).

**Abbreviations:** ER, endoplasmic reticulum; ES-TRAP, ER stress-responsive alkaline phosphatase; GRP78, 78 kDa glucose-regulated protein; CHOP, CCAAT/enhancer-binding protein-homologous protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RLU, relative light unit; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR

## 2.4. Formazan assay

The number of viable cells was assessed by a formazan assay using Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan) [7].

## 2.5. Assessment of in vivo half-life of ES-TRAP

Recombinant ES-TRAP [ $1.78 \times 10^8$  relative light unit (RLU)/500  $\mu$ l] was administered into mice intravenously (C57BL/6 mice, 25 g body weight,  $n = 5$ ), and activity of ES-TRAP in sera was evaluated after 3 min, 2, 4, 6, 8 and 24 h.

## 2.6. Generation of ES-TRAP mice

Transgenic mice were generated by microinjection of the dioxin responsive element-controlled ES-TRAP gene [11] into fertilized oocytes of C57BL/6 mice. A transgenic line constitutively producing ES-TRAP was occasionally isolated from the pool of the offspring and named ES-TRAP mice. Genomic integration of the transgene was confirmed by polymerase chain reaction (PCR) using the following primers; 5'-AACATGGACATTGACGTGATCCTAG-3' and 5'-TCTCGTATTTTCATGTCTCCAGGCTC-3'. Expression of ES-TRAP mRNA was examined by reverse transcriptase-PCR (RT-PCR) using the same primers. Expression of GAPDH mRNA was examined as an internal control using the following primers: 5'-ACCACAGTC-CATGCCATCAC-3' and 5'-TCCACCACCTGTGTCTGTA-3'.

To evaluate activity of ES-TRAP in individual organs, wild-type mice and ES-TRAP mice were perfused with PBS to wash blood out, and brains, hearts, lungs, spleens, livers and kidneys were subjected to chemiluminescent assay. Activity of ES-TRAP per 1  $\mu$ g total protein was calculated to compare the levels among different organs.

## 2.7. In vivo induction of ER stress by thapsigargin

ES-TRAP mice were administered with thapsigargin (1 mg/kg body weight; Sigma–Aldrich Japan) intraperitoneally, and after 4, 8 and 24 h, blood was sampled from the tail vein and subjected to chemiluminescent assay to evaluate serum ES-TRAP activity ( $n = 4$ ). To confirm in vivo induction of ER stress by thapsigargin, brains, hearts, lungs, livers, kidneys and spleens were sampled after 8 and 24 h, and expression of GRP78 was examined by Northern blot analysis.

## 2.8. Administration with cadmium into ES-TRAP mice

ES-TRAP mice were administered with cadmium chloride intraperitoneally (12 mg/kg body weight), and activity of serum ES-TRAP was evaluated every 2 h up to 24 h ( $n = 4$ ). To confirm in vivo induction of ER stress by cadmium, brains, hearts, lungs, spleens, livers and kidneys were sampled after 6 h, and expression of GRP78 was examined by Northern blot analysis.

## 2.9. Statistical analysis

Assays were performed in quadruplicate. Data are expressed as means  $\pm$  S.E. Statistical analysis was performed using the non-parametric Mann–Whitney  $U$  test to compare data in different groups.  $P$  value  $< 0.05$  was considered to indicate a statistically significant difference.

## 3. Results

### 3.1. Induction of ER stress in vitro by heavy metals

The kidney and liver are main targets of heavy metal-induced toxicity. We first examined whether heavy metals trigger ER stress in renal tubular epithelial cells and hepatocyte-derived cells using endogenous and exogenous indicators. NRK52E cells and Hepa-1c1c7 cells stably expressing ES-TRAP were treated with cadmium chloride, nickel acetate or cobalt chloride for 6 h, and the cells and culture media were subjected to Northern blot analysis of GRP78 and CHOP and ES-TRAP assay. As shown in Fig. 1A, dose-dependent induction of CHOP was observed in both cell types in response to any types of metals, indicating induction of ER stress. In contrast, substantial induction of GRP78, the most popular indicator of ER stress, was evident only in Hepa-1c1c7 cells exposed to 50–75  $\mu$ M cadmium, although tunicamycin induced expression of GRP78 in both cell types (Fig. 1A, right). Even under this situation, substantial suppression of ES-TRAP was observed in these cells exposed to any types of heavy metals (Fig. 1B). Of note, for the ES-TRAP studies, we used minimum concentrations of heavy metals used in Fig. 1A, some of which (nickel and cobalt) induced neither GRP78 nor CHOP in Hepa-1c1c7 cells. These results suggested that ER stress was induced by heavy metals in the cells originated from the kidney and liver and that ES-TRAP can detect metal-induced ER stress more sensitively than Northern blot analysis of endogenous biomarkers.

### 3.2. Characterization of ES-TRAP mice

To examine validity of using ES-TRAP as an in vivo indicator for ER stress, we first evaluated the half-life of ES-TRAP

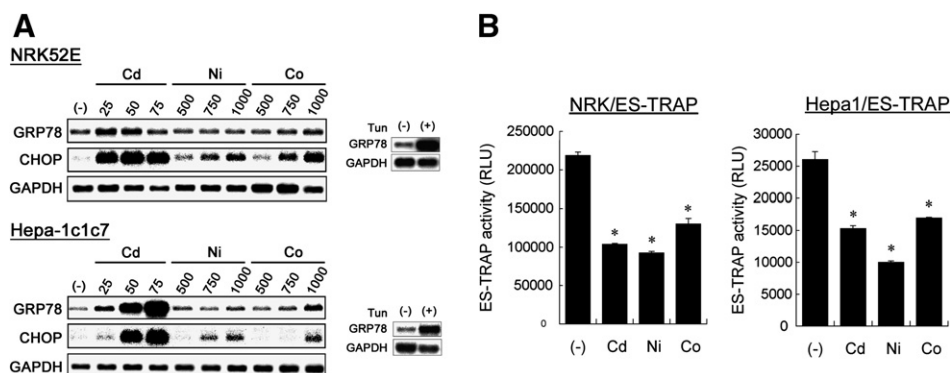


Fig. 1. In vitro induction of endoplasmic reticulum (ER) stress by heavy metals. (A) NRK52E cells and Hepa-1c1c7 cells were exposed to cadmium chloride (Cd; 25–75  $\mu$ M), nickel acetate (Ni; 500–1000  $\mu$ M), cobalt chloride (Co; 500–1000  $\mu$ M) or tunicamycin (Tun; 1  $\mu$ g/ml) for 6 h and subjected to Northern blot analysis of 78 kDa glucose-regulated protein (GRP78) and CCAAT/enhancer-binding protein-homologous protein (CHOP). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. (B) NRK/ES-TRAP cells and Hepa1/ES-TRAP cells constitutively expressing ER stress-responsive alkaline phosphatase (ES-TRAP) were exposed to 25  $\mu$ M Cd, 500  $\mu$ M Ni or 250  $\mu$ M Co for 6 h, and activity of ES-TRAP was evaluated by chemiluminescent assay. Because activity of ES-TRAP per se is modestly affected by some metals directly, especially by cadmium ( $\sim 15\%$  reduction, our observation), all values were normalized by the number of viable cells (evaluated by formazan assay) and also corrected by subtracting direct influences of metals on ES-TRAP activity. Assays were performed in quadruplicate, and data are expressed as means  $\pm$  S.E. Asterisks indicate statistically significant differences ( $P < 0.05$ ).

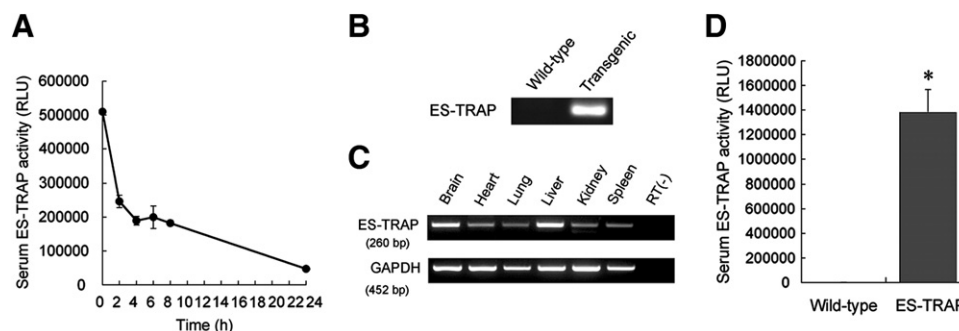


Fig. 2. Characterization of ES-TRAP mice. (A) In vivo kinetics of serum ES-TRAP activity after systemic injection of recombinant ES-TRAP in mice. Recombinant ES-TRAP protein was injected into C57BL/6 mice ( $n = 5$ ) intravenously. Serum was sampled periodically (3 min–24 h) to evaluate ES-TRAP activity. (B) Polymerase chain reaction (PCR) analysis of the ES-TRAP transgene integrated in the genome. (C) Reverse transcriptase-PCR analysis of constitutive expression of *ES-TRAP* mRNA in various organs. RT(–), reaction without reverse transcriptase. (D) Activity of serum ES-TRAP in transgenic mice. Five micro liters of sera collected from the tail veins of wild-type mice ( $n = 10$ ) and ES-TRAP mice ( $n = 10$ ) were subjected to chemiluminescent assay to evaluate activity of ES-TRAP. An asterisk indicates a statistically significant difference ( $P < 0.05$ ).

in mice. When recombinant ES-TRAP was administered into mice intravenously, the half-life of serum ES-TRAP was approximately 2 h (Fig. 2A). It suggests that altered activation, degradation or secretion of ES-TRAP caused by ER stress [7] results in rapid shift in the level of serum ES-TRAP activity. It is contrastive to secreted luciferase whose activity cannot be detected in serum and therefore unsuitable as an in vivo reporter molecule [10]. Based on this result, we established transgenic mice that produce ES-TRAP systemically. Genomic integration of the transgene was confirmed by PCR (Fig. 2B). RT-PCR analysis revealed that the ES-TRAP mice expressed *ES-TRAP* mRNA in all tested organs including brains, hearts, lungs, livers, kidneys and spleens (Fig. 2C). To confirm production of ES-TRAP protein in individual organs, wild-type mice and ES-TRAP mice were perfused with PBS to wash blood out, and tissue extracts of brains, hearts, lungs, livers, spleens and kidneys were subjected to chemiluminescent assay to evaluate ES-TRAP activity. Consistent with the result shown in Fig. 2C, activity of ES-TRAP in transgenic mice was elevated in all organs compared with wild-type mice (Table 1). In wild-type mice, the background level of serum ES-TRAP was  $7.5 \pm 1.0 \times 10^2$  RLU ( $n = 10$ ). In contrast, ES-TRAP mice exhibited high levels of serum ES-TRAP activity, approximately  $1.4 \pm 1.9 \times 10^6$  RLU ( $n = 10$ ) (Fig. 1D). ES-TRAP mice did not exhibit any abnormalities in appearances, development, fertility and life span.

### 3.3. Evidence for rapid, transient induction of ER stress in vivo after systemic exposure to heavy metal

Thapsigargin is an agent to deplete  $\text{Ca}^{2+}$  store in the ER and thereby causes ER stress. In vitro, thapsigargin induces expression of *GRP78* within several hours and rapidly suppresses ES-TRAP activity in stably transfected cells [7]. To examine a feasibility of using the ES-TRAP system for in vivo monitoring of ER stress, ES-TRAP mice were administered with thapsigargin intraperitoneally, and after 4, 8 and 24 h, blood was sampled from the tail vein and subjected to chemiluminescent assay. As shown in Fig. 3A, the serum level of ES-TRAP was significantly decreased within 4 h and reached a bottom at 8 h after the administration. The decrease in the serum ES-TRAP was recovered partially after 24 h. To confirm systemic, in vivo induction of ER stress by thapsigargin, brains, hearts, lungs,

Table 1  
Activity of ES-TRAP in individual organs of transgenic mice

Organ	ES-TRAP activity (RLU/ $\mu\text{g}$ protein)	
	Wild-type	ES-TRAP
Brain	$655 \pm 325$	$1343 \pm 168$
Heart	$237 \pm 41$	$2214 \pm 64^*$
Lung	$1383 \pm 149$	$4028 \pm 690^*$
Liver	$447 \pm 75$	$720 \pm 98^*$
Kidney	$405 \pm 98$	$1621 \pm 632$
Spleen	$266 \pm 87$	$1620 \pm 254^*$

\*  $P < 0.05$ .

livers, kidneys and spleens were sampled after 8 and 24 h, and expression of *GRP78* was examined by Northern blot analysis. The result showed that the suppression of ES-TRAP activity observed was inversely correlated with up-regulation of *GRP78*; i.e., expression of *GRP78* was induced at 8 h strongly in the liver and kidney and modestly in the lung and spleen (Fig. 3B). The expression was returned to the basal level after 24 h.

Using the established transgenic mice, we examined whether and how acute exposure to cadmium induces ER stress in its target organs. ES-TRAP mice were administered with cadmium chloride intraperitoneally, and activity of serum ES-TRAP was evaluated. As shown in Fig. 4A, activity of ES-TRAP was rapidly reduced within 2 h after the administration with cadmium. The decreased ES-TRAP activity was gradually recovered thereafter and returned to the initial level after 12 h. Consistent with this response, expression of *GRP78* was markedly induced in the liver and kidney at 6 h and recovered to the basal level at 24 h (Fig. 4B). In contrast, induction of *GRP78* was not evident in other organs including the brain, heart, lung and spleen (Fig. 4C). These results clearly showed that, after acute exposure to cadmium, ER stress is induced rapidly and predominantly in the liver and kidney. However, the induction of ER stress is transient and reversible, and quick recovery from the stress state is achieved in vivo.

## 4. Discussion

In this report, we demonstrated that heavy metals including cadmium, nickel and cobalt have the potential to induce ER

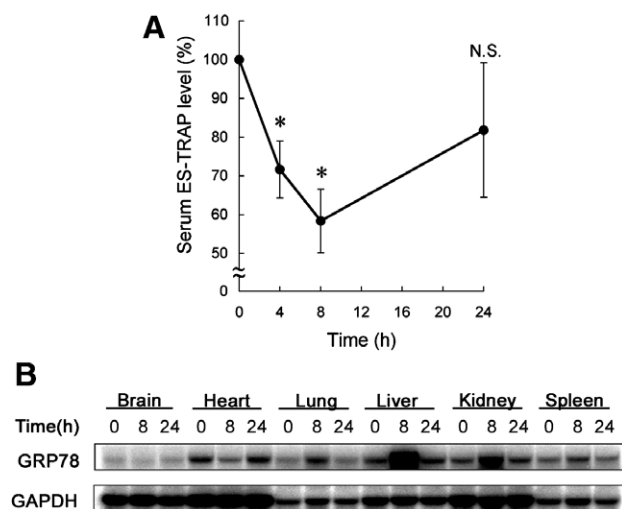


Fig. 3. Kinetics of serum ES-TRAP activity and *GRP78* expression after thapsigargin-induced, systemic ER stress. (A) ES-TRAP mice ( $n = 4$ ) were injected with thapsigargin (1 mg/kg body weight) intraperitoneally. Serum was sampled periodically after 4, 8 and 24 h and subjected to chemiluminescent assay. Data (relative percentages vs. initial level) are expressed as means  $\pm$  S.E. Asterisks indicate statistically significant differences ( $P < 0.05$ ). N.S., not statistically significant. (B) ES-TRAP mice were administered with thapsigargin intraperitoneally, and after 8 and 24 h, indicated organs were subjected to Northern blot analysis of *GRP78*.

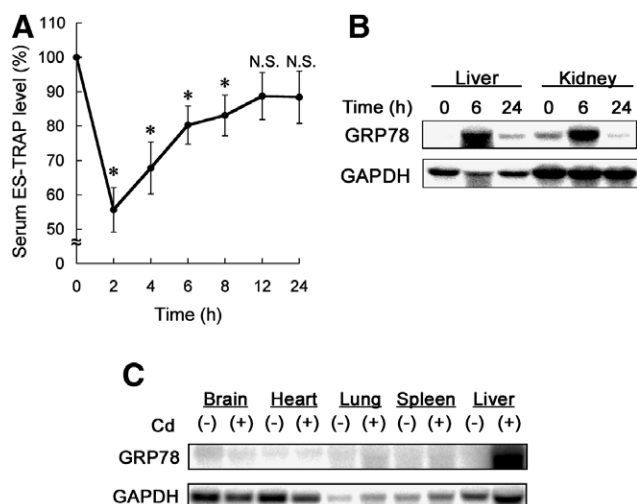


Fig. 4. Real-time monitoring of ER stress in vivo after acute exposure to cadmium. (A) Kinetics of serum ES-TRAP activity. ES-TRAP mice ( $n = 4$ ) were injected with cadmium chloride intraperitoneally (12 mg/kg body weight), and serum was sampled periodically and subjected to chemiluminescent assay. Data (relative percentages vs. initial level) are expressed as means  $\pm$  S.E. Asterisks indicate statistically significant differences ( $P < 0.05$ ). N.S., not statistically significant. (B) Northern blot analysis of *GRP78* in the liver and kidney. ES-TRAP mice were injected with cadmium chloride intraperitoneally, and after 6 h and 24 h, the livers and kidneys were subjected to Northern blot analysis. (C) Northern blot analysis of *GRP78* in other organs. Six hours after the administration with cadmium, indicated organs were sampled and subjected to Northern blot analysis.

stress in renal proximal tubular cells and hepatocyte-derived cells. We also showed that acute, systemic exposure of mice to cadmium caused rapid, transient and reversible induction

of ER stress in vivo, especially in the liver and kidney. Previous investigation showed that exposure to cadmium led to accumulation of this metal in the liver and kidney and caused renal tubular and hepatic injury [2,12,13]. Several reports indicated that toxic effects of cadmium were mediated by oxidative stress and mitochondrial dysfunction [13,14]. However, to date, roles of ER stress in cadmium-induced tissue injury have not been reported. Our current results suggested a novel mechanism that mediates cadmium-induced organ dysfunction. Of note, the induction of ER stress by heavy metals was observed not only by cadmium but also by other toxic metals. It indicates a possibility that the similar mechanism may also participate in other heavy metal-induced tissue injury.

In the present report, we demonstrated the usefulness of ES-TRAP as an in vivo indicator for ER stress using transgenic mice. The ES-TRAP-based sensing system has several advantages for in vivo monitoring of ER stress. First and foremost, activity of ES-TRAP can be quantified quickly and sensitively by using conventional chemiluminescent systems, and only 5  $\mu$ l of serum is sufficient for the assessment. It allows for non-invasive, real-time assessment of ER stress in vivo during the course of diseases. If mice transgenic with the ES-TRAP gene under the control of tissue- or cell type-specific promoters are generated, monitoring of ER stress in particular tissues or specific organs will be feasible by simple blood sampling. Recently, Iwawaki and co-workers reported monitoring for ER stress using a variant of *XBP-1* mRNA as a sensor [15]. However, by this approach, successive in vivo monitoring of ER stress may be difficult, because tissue sampling and/or exposure of internal organs are inevitable for evaluation of fluorescence.

Monitoring of ER stress is required for investigation of a broad range of pathophysiological events in vivo. However, conventional approaches require biopsy of local tissues and extraction of protein or RNA to evaluate the level of endogenous biomarkers. To our knowledge, our system described here is the first that allows for non-invasive, real-time monitoring of ER stress in vivo. The ES-TRAP method would be useful for: (1) elucidation of novel roles of ER stress in various pathophysiological situations, (2) monitoring for activity of ER stress-related diseases and evaluation of therapeutic effectiveness, (3) screening for adverse effects of drugs that causes ER stress, and (4) development of therapeutic agents for prevention and attenuation of ER stress-associated disorders.

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